A NEW BLOOD-COUNTING PIPETTE, FOR ESTIMATING THE NUMBERS OF LEUCOCYTES AND BLOOD PARASITES PER CUBIC MILLIMETRE

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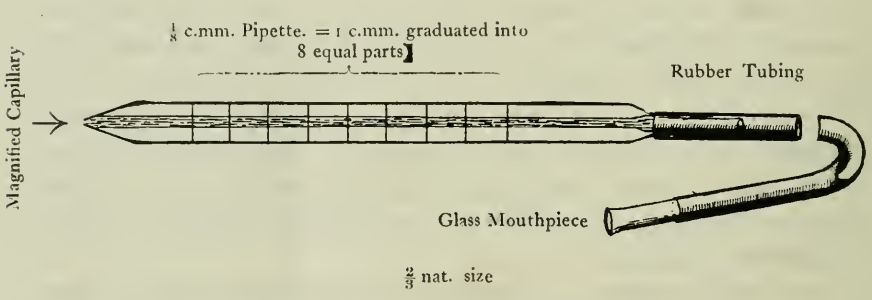
Prefatory Note.—This pipette* has been devised to facilitate an extensive research, carried on during the past two years, on the exact enumeration of parasites in the blood of cases of malarial fever and sleeping sickness. The research was instigated and directed by Sir Ronald Ross, K.C.B., F.R.S., and conducted with the aid of funds supplied by the Advisory Committee of the Colonial Office and a private fund given by Sir Edwin Durning-Lawrence in connection with cryotherapy, at the Liverpool School of Tropical Medicine, and at the Tropical Laboratory in the Royal Southern Hospital, Liverpool, and has revealed a number of facts in both these diseases.

We first attempted to enumerate the parasites in malarial fever by finding the ratio of leucocytes to parasites in dehaemoglobinised thick blood films (Ross, 1903). This method, however, proved to be tedious and inaccurate, as it involved the double process of making a leucocyte count with a Thoma Zeiss, and finding the leucocyte-parasite ratio in a thick blood preparation. Later, at the suggestion of Sir Ronald Ross, I commenced to count the parasites directly in a measured sample of blood, blown from a graduated capillary pipette and made into a thick film. This latter method gave more accurate results, and, finally, by devising a special pipette, I was able to enumerate quickly and with considerable accuracy both leucocytes and parasites simultaneously in the specimen. The references to the literature of the researches carried on by this method will be found at the end of this paper.

* The pipettes are sold by C. Baker, 244 High Holborn, London, W.C., Price 10/6 each.

I. DESCRIPTION OF THE PIPETTE

The pipette is about five inches long, and is made of glass tubing similar to that used for thermometers, with a white opal background and powerful magnifying surface. The capillary bore, however, is not flattened out like that of a thermometer but is more nearly circular in cross section. The bore is exceedingly fine and hair-like—such that a portion of the capillary about 60 to 70 millimetres long has a capacity of one cubic millimetre. This cubic millimetre length is graduated into equal parts, so that a given fraction of a cubic millimetre of blood can be accurately blown out. A rubber tube with a glass mouthpiece is attached to one end of the pipette, and the other end tapers to a very fine point—which is necessary for expelling accurate small droplets of blood (see diagram).



2/3 nat. size

The pipette possesses about the limit of capillary fineness for blood work, because in using a capillary of less diameter it is difficult to expel the blood (which clots quickly in so fine a bore). When this clotting occurs it is troublesome to clear the pipette, as there is no wire fine enough or rigid enough to penetrate it. Even with the bore employed, considerable care and practice are required; but in experienced hands the pipette may be used constantly for months without any blockage occurring. In view of the above difficulty, I have devised a similar pipette with a larger bore and graduated to $\frac{1}{4}$ c.mm. This latter can be easily penetrated by a fine wire and cleaned in case of a stoppage. Such a pipette (of the larger bore) may be preferred by beginners; but for quicker work and for those who have had some practice, the $\frac{1}{8}$ c.mm. pipette is the better.

II. METHOD OF USING THE PIPETTE

(1) Prick the ear or finger, and allow a tiny droplet of blood to exude. Do not squeeze (or only very little), as squeezing drives out lymph and lymphocytes from the lymphatics.

(2) Suck the blood into the pipette.

(3) Expel the blood until the column coincides with a line on the magnifying surface. Wipe the point of the pipette and expel $\frac{1}{8}$ or $\frac{1}{4}$ c.mm. of blood on to a clean glass slide. After this has been carefully done, expel all the blood immediately from the pipette, otherwise it will clot in the bore.

(4) Breathe on the measured droplet of blood on the slide, and spread it by means of the point of the pricking needle into a small square, about 4 mm. × 4 mm. This square should be spread as uniformly and as neatly as possible, and takes a little practice. A fine triangular pointed needle serves the purpose best. A certain amount of breathing on the slide is essential to keep the blood from drying up during the spreading process. In the case of a $\frac{1}{4}$ c.mm. droplet, the spread square should be a little larger—about 5 mm. × 5 mm.

(5) Allow the little square blood film to dry in air. This takes place in a few seconds. Fix for about two minutes in absolute alcohol and stain with the usual blood stains. In the case of Jenner's or Leishman's stain, previous fixing is of course unnecessary. Wash gently and dry on blotting paper.

(6) If it is desired to enumerate asexual malaria parasites, the square film should be spread over a smaller area into a thicker film and fixed in acid alcohol (5 per cent. dilute acetic acid in absolute alcohol) before staining. The corpuscles are dehaemoglobinised by this procedure and do not obscure the parasites. To count leucocytes, trypanosomes and crescents this dehaemoglobinisation is unnecessary.

(7) The pipette should be cleaned and dried immediately after use. This is done by sucking up water and expelling it alternately several times. Repeat this process with absolute alcohol or ether, and finally suck air through to dry the bore. By watching the bore through the magnifying surface one can tell when it is dry. The water and alcohol used for cleansing and drying should be free from dust.

III. HOW TO CLEAN THE PIPETTE IN CASE OF STOPPAGE

If the blood should clot in the pipette, it can often be expelled by moistening the point of the pipette with water or by forcing water through with a Higginson's syringe. If this fails it may be cleaned as follows. Heat the pipette in the Bunsen flame gradually, but do not heat the point. Now immerse the point in strong nitric acid, and allow to stand there till it cools. This enables the nitric acid to get into the bore. Dry the outside of the pipette; press a piece of indiarubber firmly against the point and at the same time heat it with the Bunsen flame as near to the point as possible. This procedure produces vaporised nitric acid in the bore under high pressure. The vapour eats away the clot and drives it upwards. The process may have to be repeated several times if the clot is a large one. The clot may also be slowly dissolved by completely immersing the pipette in a test-tubeful of nitric acid. Place the test-tube in a beaker of water and alternately boil and cool. This latter is a slower but safer cleansing procedure. In the first method the pipette must be dry and heated gradually, otherwise it may crack.

Sometimes the point only of the pipette becomes blocked with material other than blood. It can easily be cleared by keeping the point immersed in strong nitric acid for some time.

When the pipette is in constant use, it is a good practice to suck strong nitric acid into the bore and allow it to remain there all night. This cleans away any blood or serum which may have commenced to accumulate in the bore or at the orifice. Before using the pipette one should blow through it into alcohol to see if the air bubbles through freely; as the most frequent cause of blocking is an attempt to suck up and expel blood through a partially obstructed orifice. The $\frac{1}{4}$ c.mm. pipette can be cleaned out by means of a fine wire.*

IV. METHOD OF ENUMERATING LEUCOCYTES OR PARASITES IN THE MEASURED SQUARE BLOOD FILM

A microscope with a mechanical sliding stage and an eyepiece having a diaphragm with a square hole is essential. For the latter

* For the pepsin method of cleaning pipettes, see Stephens & Christophers, 'Practical Study of Malaria,' third edition, page 11.

a circle of paper with a square hole may be fitted into the eyepiece; but an Ehrlich's ocular eyepiece is better, as by means of this eyepiece the square microscopic field can be contracted by means of a little lever to any size required.

Place a drop of oil on the square blood film, without a cover-glass, and place under oil immersion lens. Find the upper margin of the square film, and by means of the mechanical stage work down, field by field, towards the lower margin. On reaching the fifth field from the upper margin, stop, and move the field from the right margin of the square film to the left margin, meanwhile counting the number of leucocytes or parasites all the way in one imaginary band the breadth of the microscopic field. Now move down to the tenth band from the top margin and repeat this process, then move down to the fifteenth band and repeat again, and finally move down to the lower margin.

Let us suppose that the number of microscopic field diameters between the upper and lower margins is 30, and that the average number of leucocytes or parasites in the three bands counted is 40, then the total number in the square film is $30 \times 40 = 1200$. But the square film represents $\frac{1}{8}$ c.mm., therefore the number per c.mm. is $8 \times 1200 = 9600$. If the square film is $\frac{1}{4}$ c.mm., then the number will be, of course, 4800 per c.mm.

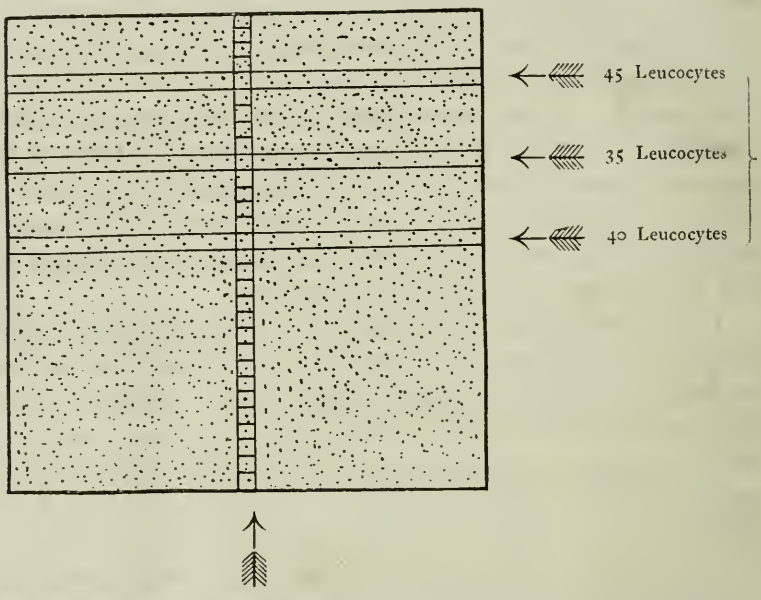
The diagram on page 476 may indicate the method more clearly.

The greater the number of bands counted, the greater is the accuracy of the result; but in a well-spread film where the distribution of leucocytes or parasites is fairly uniform, a count of three bands will be amply sufficient, but one must count a varying number of bands according to the number of parasites or leucocytes present. Where the parasites are scarce, say under 1000 or 2000 per c.mm. (crescents are nearly always below this number), then it is not sufficiently accurate to calculate the number from bands, and the total number in the whole of the square film must be counted by means of the mechanical stage. Where the numbers are very small, it may be necessary to take a larger specimen of blood than $\frac{1}{8}$ or $\frac{1}{4}$ c.mm. The examination of the whole square takes about ten minutes if the objects counted are few.

Special slides can be obtained having a square 4 mm. × 4 mm. ruled on the glass. This enables one to spread the droplet of blood

into an accurate square, and by finding the number of diameters of the microscopic field across this ruled square, one obtains a constant multiplying factor for estimating the number of leucocytes, etc., in blood films spread exactly over that square.

Square Blood Film $\frac{1}{8}$ c.mm.



The side of the Total Square Film is 30 times the diameter of the Square Microscopic Field.

Average number of leucocytes is 40 in a band the breadth of microscopic field.

$\therefore$ $40 \times 30 \times 8 = 9600$ leucocytes per c.mm.

V. SOURCES OF ERROR IN THE METHOD

I do not claim to have calculated the amount of error in this method mathematically, but so far as I can estimate, the errors are as follows:—

(1) With this pipette I estimate that the instrumental error in transferring $\frac{1}{8}$ c.mm. of blood to a slide is 5 per cent.

(2) Where only a few bands are counted across the square, there is an error due to unequal spread of the film. The greater the number of bands counted the smaller is the error, and where the whole of the square is counted this source of error is eliminated altogether.

(3) The error depending upon the number of objects counted has been fully discussed by Sir Ronald Ross and Mr. Walter Stott (1911). By referring to tables in their article, one can tell the exact percentage of error for the number of objects counted. It appears that to get within a statistical error of 5 per cent. one must count at least 200 objects. (For further details see Section IV of their article.)

VI. COMPARISON WITH THE THOMA-ZEISS METHOD

The following table gives a comparison between leucocyte counts made simultaneously by this method and by the Thoma-Zeiss apparatus: —

Case	Thoma-Zeiss	New method (average of two bands only)
1	27,000 leucocytes per c.mm.	26,000 leucocytes per c.mm.
2	15,000 ,, ,,	16,000 ,, ,,
3	14,000 ,, ,,	12,400 ,, ,,
4	16,000 ,, ,,	15,500 ,, ,,
5	13,000 ,, ,,	13,600 ,, ,,
6	40,000 ,, ,,	43,000 ,, ,,
7	10,000 ,, ,,	10,200 ,, ,,
8	17,000 ,, ,,	15,100 ,, ,,

VII. ADVANTAGES OF THE NEW METHOD

(1) Blood parasites can be enumerated. The parasites of malaria and sleeping sickness cannot be enumerated accurately by any other means.

(2) Only a tiny droplet of blood is required. This renders the method very suitable for those who desire to investigate the blood of small animals such as rats, mice, etc. It is also important where frequent examinations are being made on one patient. The patient does not object to the gentle prick of the needle nor to the small amount of blood taken.

(3) No diluting fluid or special slide is required.

(4) The slides with the required square films can be stored,

stained and counted at any future time when convenient. A large number of samples can thus be taken at frequent intervals and these can be kept and examined at a later date.

(5) A differential leucocyte count can be made simultaneously with the enumeration.

(6) Auto-agglutination of the red cells can be detected through the magnifying surface of the capillary.

(7) After some practice the method will be found to be extremely rapid and convenient.

I am indebted to Dr. J. J. Levin who kindly made the Thoma-Zeiss counts in the above table.

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